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METHOD FOR LINEAR mRNA AMPLIFICATION

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INTRODUCTION

<u>Technical Field</u>

The technical field of this invention is the enzymatic amplification of nucleic

15 acids.

<u>Background of the Invention</u>

The characterization of cellular gene expression finds application in a variety
of disciplines, such as in the analysis of differential expression between different tissue
20 types, different stages of cellular growth or between normal and diseased states.
Fundamental to differential expression analysis is the detection of different mRNA
species in a test population, and the quantitative determination of different mRNA
levels in that test population. However, the detection of rare mRNA species is often
complicated by one or more of the following factors: cell heterogeneity, paucity of
25 material, or the limits of detection of the assay method. Thus, methods which amplify
heterogeneous populations of mRNA that do not introduce significant changes in the
relative amounts of different mRNA species facilitate this technology.

A number of methods for the amplification of nucleic acids have been
described. Such methods include the "polymerase chain reaction" (PCR) (Mullis *et*
30 *al.*, United States Patent Number 4,683,195), and a number of transcription-based
amplification methods (Malek *et al.*, U.S. Patent No. 5,130,238; Kacian and Fultz,
U.S. Patent No. 5,399,491; Burg *et al.*, U.S. Patent No. 5,437, 990). Each of these
methods uses primer-dependent nucleic acid synthesis to generate a DNA or RNA
product, which serves as a template for subsequent rounds of primer-dependent
35 nucleic acid synthesis. Each process uses (at least) two primer sequences
complementary to different strands of a desired nucleic acid sequence and results in an
exponential increase in the number of copies of the target sequence. These
amplification methods can provide enormous amplification (up to billion-fold).

1

However, these methods have limitations which make them not amenable for gene expression monitoring applications. First, each process results in the specific amplification of only the sequences that are bounded by the primer binding sites. Second, exponential amplification can introduce significant changes in the relative amounts of specific target species--small differences in the yields of specific products (for example, due to differences in primer binding efficiencies or enzyme processivity) become amplified with every subsequent round of synthesis.

Amplification methods that utilize a single primer are amenable to the amplification of heterogeneous mRNA populations. The vast majority of mRNAs carry a homopolymer of 20-250 adenosine residues on their 3' ends (the poly-A tail), and the use of poly-dT primers for cDNA synthesis is a fundamental tool of molecular biology. "Single-primer amplification" protocols have been reported (see e.g. Kacian *et al.*, U.S. Patent No. 5,554,516; Van Gelder *et al.*, U.S. Patent No. 5,716,785). The methods reported in these patents utilize a single primer containing an RNA polymerase promoter sequence and a sequence complementary to the 3'-end of the desired nucleic acid target sequence(s) ("promoter-primer"). In both methods, the promoter-primer is added under conditions where it hybridizes to the target sequence(s) and is converted to a substrate for RNA polymerase. In both methods, the substrate intermediate is recognized by RNA polymerase, which produces multiple copies of RNA complementary to the target sequence(s) ("antisense RNA"). Each method uses, or could be adapted to use, a primer containing poly-dT for amplification of heterogeneous mRNA populations.

Amplification methods that proceed linearly during the course of the amplification reaction are less likely to introduce bias in the relative levels of different mRNAs than those that proceed exponentially. In the method described in U.S. Patent No. 5,554,516, the amplification reaction contains a nucleic acid target sequence, a promoter-primer, an RNA polymerase, a reverse transcriptase, and reagent and buffer conditions sufficient to allow amplification. The amplification proceeds in a single tube under conditions of constant temperature and ionic strength. Under these conditions, the antisense RNA products of the reaction can serve as substrates for further amplification by non-specific priming and extension by the RNA-dependent DNA polymerase activity of reverse transcriptase. As such, the amplification described in U.S. Patent No. 5,554,516 proceeds exponentially. In contrast, in specific examples described in U.S. Patent No. 5,716,785, cDNA synthesis and transcription
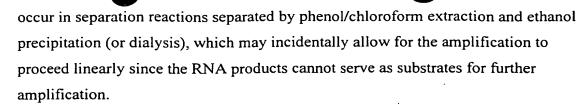
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occur in separation reactions separated by phenol/chloroform extraction and ethanol precipitation (or dialysis), which may incidentally allow for the amplification to proceed linearly since the RNA products cannot serve as substrates for further amplification.

5   The method described in U.S. Patent No. 5,716,785 has been used to amplify cellular mRNA for gene expression expression monitoring (for example, R.N. Van Gelder *et al.* (1990), Proc. Natl. Acad. Sci. USA 87, 1663; D.J. Lockhart *et al.* (1996), Nature Biotechnol. 14, 1675). However, this procedure is not readily amenable to high throughput processing. In preferred embodiments of the method described in

10   U.S. Patent No. 5,716,785, poly-A mRNA is primed with a promoter-primer containing poly-dT and converted into double-stranded cDNA using a method described by Gubler and Hoffman (U. Gubler and B.J. Hoffman (1983), Gene 25, 263-269) and popularized by commercially available kits for cDNA synthesis. Using this method for cDNA synthesis, first strand synthesis is performed using reverse

15   transcriptase and second strand cDNA is synthesized using RNaseH and DNA polymerase I. After phenol/chloroform extraction and dialysis, double-stranded cDNA is transcribed by RNA polymerase to yield antisense RNA product. The phenol/chloroform extractions and buffer exchanges required in this procedure are labor intensive, and are not readily amenable to robotic handling.

20   Accordingly, there is interest in the development of improved methods of antisense RNA amplification. Of particular interest would be the development of a linear amplification protocol that did not require a reverse transcriptase separation step.

Relevant Literature

25   United States Patents disclosing methods of antisense RNA synthesis include: 5,716,785; 5,554,516; 5,545,522; 5,437,990; 5,130,238; and 5,514,545. Antisense RNA synthesis is also discussed in Phillips and Eberwine (1996), Methods: A companion to Methods in Enzymol. 10, 283; Eberwine *et al.* (1992), Proc., Natl., Acad. Sci. USA 89, 3010; Eberwine (1996), Biotechniques 20, 584; and Eberwine *et*

30   *al.* (1992), Methods in Enzymol. 216, 80.

SUMMARY OF THE INVENTION

Methods for linearly amplifying mRNA to produce RNA (particularly as antisense RNA) are provided. In the subject methods, mRNA is converted to cDNA

3

(particularly double-stranded cDNA) using a promoter-primer particularly having a poly-dT primer site linked to a promoter sequence so that the resulting cDNA is recognized by an RNA polymerase. The resultant cDNA is then transcribed into RNA (particularly antisense RNA) in the presence of a reverse transcriptase that is rendered incapable of RNA-dependent DNA polymerase activity during this transcription step. The subject methods find use a variety of different applications in which the preparation of linearly amplified amounts of antisense RNA is desired. Also provided are kits for practicing the subject methods.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 provides a graphical representation of the results obtained using the subject methods versus those described in U.S. Patent No. 5,716,785.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods for linearly amplifying mRNA to produce antisense RNA are provided. In the subject methods, mRNA is converted to double-stranded cDNA using a promoter-primer having a poly-dT primer site linked to a promoter sequence so that the resulting double-stranded cDNA is recognized by an RNA polymerase. The resultant double-stranded cDNA is then transcribed into antisense RNA in the presence of a reverse transcriptase that is rendered incapable of RNA-dependent DNA polymerase activity during this transcription step. The subject methods find use in a variety of different applications in which the preparation of linearly amplified amounts of antisense RNA is desired. Also provided are kits for practicing the subject methods.

Before the subject invention is described further, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as variations of the particular embodiments may be made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended to be limiting. Instead, the scope of the present invention will be established by the appended claims.

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It must be noted that as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

The subject invention provides methods for linearly amplifying mRNA into antisense RNA. As such, the subject invention provides methods of producing amplified amounts of antisense RNA from an initial amount of mRNA. By amplified amounts is meant that for each initial mRNA, multiple corresponding antisense RNAs, where the term antisense RNA is defined here as ribonucleic acid complementary to the initial mRNA, are produced. By corresponding is meant that the antisense RNA shares a substantial amount of sequence identity with the sequence complementary to the mRNA (*i.e.* the complement of the initial mRNA), where substantial amount means at least 95% usually at least 98% and more usually at least 99%, where sequence identity is determined using the BLAST algorithm, as described in Altschul *et al.* (1990), J. Mol. Biol. 215:403-410 (using the published default setting, *i.e.* parameters $w = 4$, $t = 17$). Generally, the number of corresponding antisense RNA molecules produced for each initial mRNA during the subject linear amplification methods will be at least about 10, usually at least about 50 and more usually at least about 100, where the number may be as great as 600 or greater, but often does not exceed about 1000.

In the first step of the subject methods, an initial mRNA sample is subjected to a series of enzymatic reactions under conditions sufficient to ultimately produce double-stranded DNA for each initial mRNA in the sample that is amplified. During this first step, an RNA polymerase promoter region is incorporated into the resultant product, which region is critical for the second step of the subject methods, *i.e.* the transcription step described in greater detail *infra*.

The initial mRNA may be present in a variety of different samples, where the sample will typically be derived from a physiological source. The physiological source may be derived from a variety of eukaryotic sources, with physiological sources of interest including sources derived from single-celled organisms such as yeast and multicellular organisms, including plants and animals, particularly mammals, where the physiological sources from multicellular organisms may be

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derived from particular organs or tissues of the multicellular organism, or from isolated cells derived therefrom. In obtaining the sample of RNA to be analyzed from the physiological source from which it is derived, the physiological source may be subjected to a number of different processing steps, where such processing steps might

5   include tissue homogenization, cell isolation and cytoplasm extraction, nucleic acid extraction and the like, where such processing steps are known to those of skill in the art. Methods of isolating RNA from cells, tissues, organs or whole organisms are known to those of skill in the art and are described in Maniatis *et al.* (1989), Molecular Cloning: A Laboratory Manual 2d Ed. (Cold Spring Harbor Press). Alternatively, at

10   least some of the initial steps of the subject methods may be performed *in situ*, as described in U.S. Patent No. 5,514,545, the disclosure of which is herein incorporated by reference.

Depending on the nature of the primer employed during first strand synthesis, as described in greater detail below, the subject methods can be used to produce

15   amplified amounts of antisense RNA corresponding to substantially all of the mRNA present in the initial sample, or to a proportion or fraction of the total number of distinct mRNAs present in the initial sample. By substantially all of the mRNA present in the sample is meant more than 90%, usually more than 95%, where that portion not amplified is solely the result of inefficiencies of the reaction and not

20   intentionally excluded from amplification.

The promoter-primer employed in the amplification reaction includes: (a) a poly-dT region for hybridization to the poly-A tail of the mRNA; and (b) an RNA polymerase promoter region 5' of the –poly-dT region that is in an orientation capable of directing transcription of antisense RNA. In certain embodiments, the primer will

25   be a "lock-dock" primer, in which immediately 3' of the poly-dT region is either a "G', "C", or "A" such that the primer has the configuration of 3'-XTTTTTTTT.....5', where X is "G", "C", or "A". The poly-dT region is sufficiently long to provide for efficient hybridization to the poly-A tail, where the region typically ranges in length from 10-50 nucleotides in length, usually 10-25 nucleotides in length, and more

30   usually from 14 to 20 nucleotides in length.

A number of RNA polymerase promoters may be used for the promoter region of the first strand cDNA primer, *i.e.* the promoter-primer. Suitable promoter regions will be capable of initiating transcription from an operationally linked DNA sequence in the presence of ribonucleotides and an RNA polymerase under suitable conditions.

6

The promoter will be linked in an orientation to permit transcription of antisense RNA. A linker oligonucleotide between the promoter and the DNA may be present, and if, present, will typically comprise between about 5 and 20 bases, but may be smaller or larger as desired. The promoter region will usually comprise between about 15 and

5   250 nucleotides, preferably between about 17 and 60 nucleotides, from a naturally occurring RNA polymerase promoter or a consensus promoter region, as described in Alberts *et al.* (1989) *in* Molecular Biology of the Cell, 2d Ed. (Garland Publishing, Inc.). In general, prokaryotic promoters are preferred over eukaryotic promoters, and phage or virus promoters most preferred. As used herein, the term "operably linked"

10   refers to a functional linkage between the affecting sequence (typically a promoter) and the controlled sequence (the mRNA binding site). The promoter regions that find use are regions where RNA polymerase binds tightly to the DNA and contain the start site and signal for RNA synthesis to begin. A wide variety of promoters are known and many are very well characterized. Representation promoter regions of particular

15   interest include T7, T3 and SP6 as described in Chamberlin and Ryan, The Enzymes (*ed.* P. Boyer, Academic Press, New York) (1982) pp 87-108.

Where one wishes to amplify only a portion of the mRNA species in the sample, one may optionally provide for a short arbitrary sequence 3' of the poly- dT region, where the short arbitrary sequence will generally be less than 5 nucleotides in

20   length and usually less than 2 nucleotides in length, where the dNTP immediately adjacent to the poly-dT region will not be a T residue and usually the sequence will comprise no T residue. Such short 3' arbitrary sequences are described in Ling and Pardee (1992), Science 257, 967.

The promoter-primer described above and throughout this specification may be

25   prepared using any suitable method, such as, for example, the known phosphotriester and phosphite triester methods, or automated embodiments thereof. In one such automated embodiment, dialkyl phosphoramidites are used as starting materials and may be synthesized as described by Beaucage *et al.* (1981), Tetrahedron Letters 22, 1859. One method for synthesizing oligonucleotides on a modified solid support is

30   described in U.S. Patent No. 4,458,066. It is also possible to use a primer that has been isolated from a biological source (such as a restriction endonuclease digest). The primers herein are selected to be "substantially" complementary to each specific sequence to be amplified, *i.e.;* the primers should be sufficiently complementary to hybridize to their respective targets. Therefore, the primer sequence need not reflect

7

the exact sequence of the target, and can, in fact be "degenerate." Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the target to be amplified to permit hybridization and extension.

5   In the first step of the subject method, the oligonucleotide promoter-primer is hybridized with an initial mRNA sample and the primer-mRNA hybrid is converted to a double-stranded cDNA product that is recognized by an RNA polymerase. The promoter-primer is contacted with the mRNA under conditions that allow the poly-dT site to hybridize to the poly-A tail present on most mRNA species. The catalytic

10   activities required to convert primer-mRNA hybrid to double-stranded cDNA are an RNA-dependent DNA polymerase activity, a RNaseH activity, and a DNA-dependent DNA polymerase activity. Most reverse transcriptases, including those derived from Moloney murine leukemia virus (MMLV-RT), avian myeloblastosis virus (AMV-RT), bovine leukemia virus (BLV-RT), Rous sarcoma virus (RSV) and human

15   immunodeficiency virus (HIV-RT) catalyze each of these activities. These reverse transcriptases are sufficient to convert primer-mRNA hybrid to double-stranded DNA in the presence of additional reagents which include, but are not limited to: dNTPs; monovalent and divalent cations, e.g. KCl, $MgCl_2$; sulfhydryl reagents, e.g. dithiothreitol; and buffering agents, e.g. Tris-Cl. Alternatively, a variety of proteins

20   that catalyze one or two of these activities can be added to the cDNA synthesis reaction. For example, MMLV reverse transcriptase lacking RNaseH activity (described in U.S. Patent No. 5,405,776) which catalyzes RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity, can be added with a source of RNaseH activity, such as the RNaseH purified from cellular sources,

25   including *Escherichia coli*. These proteins may be added together during a single reaction step, or added sequentially during two or more substeps. Finally, additional proteins that may enhance the yield of double-stranded DNA products may also be added to the cDNA synthesis reaction. These proteins include a variety of DNA polymerases (such as those derived from *E. coli*, thermophilic bacteria, archaebacteria,

30   phage, yeasts, Neurosporas, Drosophilas, primates and rodents), and DNA Ligases (such as those derived from phage or cellular sources, including T4 DNA Ligase and *E. coli* DNA Ligase).

Conversion of primer-mRNA hybrid to double-stranded cDNA by reverse transcriptase proceeds through an RNA:DNA intermediate which is formed by
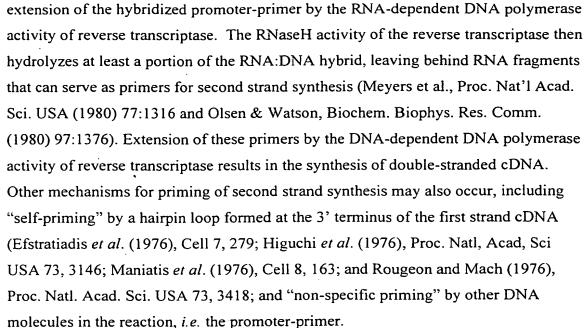
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extension of the hybridized promoter-primer by the RNA-dependent DNA polymerase activity of reverse transcriptase. The RNaseH activity of the reverse transcriptase then hydrolyzes at least a portion of the RNA:DNA hybrid, leaving behind RNA fragments that can serve as primers for second strand synthesis (Meyers et al., Proc. Nat'l Acad. Sci. USA (1980) 77:1316 and Olsen & Watson, Biochem. Biophys. Res. Comm. (1980) 97:1376). Extension of these primers by the DNA-dependent DNA polymerase activity of reverse transcriptase results in the synthesis of double-stranded cDNA. Other mechanisms for priming of second strand synthesis may also occur, including "self-priming" by a hairpin loop formed at the 3' terminus of the first strand cDNA (Efstratiadis *et al.* (1976), Cell 7, 279; Higuchi *et al.* (1976), Proc. Natl, Acad, Sci USA 73, 3146; Maniatis *et al.* (1976), Cell 8, 163; and Rougeon and Mach (1976), Proc. Natl. Acad. Sci. USA 73, 3418; and "non-specific priming" by other DNA molecules in the reaction, *i.e.* the promoter-primer.

The second strand cDNA synthesis results in the creation of a double-stranded promoter region. The second strand cDNA includes not only a sequence of nucleotide residues that comprise a DNA copy of the mRNA template, but also additional sequences at its 3' end which are complementary to the promoter-primer used to prime first strand cDNA synthesis. The double-stranded promoter region serves as a recognition site and transcription initiation site for RNA polymerase, which uses the second strand cDNA as a template for multiple rounds of RNA synthesis during the next stage of the subject methods.

Depending on the particular protocol, the same or different DNA polymerases may be employed during the cDNA synthesis step. In a preferred embodiment, a single reverse transcriptase, most preferably MMLV-RT, is used as a source of all the requisite activities necessary to convert primer-mRNA hybrid to double-stranded cDNA. In another preferred embodiment, the polymerase employed in first strand cDNA synthesis is different from that which is employed in second strand cDNA synthesis. Specifically, a reverse transcriptase lacking RNaseH activity (e.g. Superscript II [TM]) is combined with the primer-mRNA hybrid during a first substep for first strand synthesis. A source of RNaseH activity, such as *E. coli* RNaseH or MMLV-RT, but most preferably MMLV-RT, is added during a second substep to initiate second strand synthesis. In yet other embodiments, the requisite activities are provided by a plurality of distinct enzymes. The manner is which double-stranded cDNA is produced from the initial mRNA is not critical to certain embodiments of the

invention. However, the preferred embodiments use MMLV-RT, or a combination of Superscript II™ and MMLV-RT, or a combination of Superscript II™ and *E. coli* RNaseH, for cDNA synthesis as these embodiments yield certain desired results. Specifically, in the preferred embodiments, reaction conditions were chosen so that enzymes present during the cDNA synthesis do not adversely affect the subsequent transcription reaction. Potential inhibitors include, but are not limited to, RNase contaminants of certain enzyme preparations.

The next step of the subject method is the preparation of antisense RNA from the double-stranded cDNA prepared in the first step. During this step, the double-stranded cDNA produced in the first step is transcribed by RNA polymerase to yield antisense RNA, which is complementary to the initial mRNA target from which it is amplified. A critical feature of the invention is that this second step is carried out in the presence of reverse transcriptase which is present in the reaction mixture from the first step. Thus, the subject methods do not involve a step in which the double-stranded cDNA is physically separated from the reverse transcriptase following double-stranded cDNA preparation. Critical to the subject methods is that the reverse transcriptase that is present during the transcription step is rendered inactive. Thus, the transcription step is carried out in the presence of a reverse transcriptase that is unable to catalyze RNA-dependent DNA polymerase activity, at least for the duration of the transcription step. As a result, the antisense RNA products of the transcription reaction cannot serve as substrates for additional rounds of amplification, and the amplification process cannot proceed exponentially.

The reverse transcriptase present during the transcription step may be rendered inactive using any convenient protocol. The transcriptase may be irreversibly or reversibly rendered inactive. Where the transcriptase is reversibly rendered inactive, the transcriptase is physically or chemically altered so as to no longer able to catalyze RNA-dependent DNA polymerase activity. The transcriptase may be irreversibly inactivated by any convenient means. Thus, the reverse transcriptase may be heat inactivated, in which the reaction mixture is subjected to heating to a temperature sufficient to inactivate the reverse transcriptase prior to commencement of the transcription step. In these embodiments, the temperature of the reaction mixture and therefore the reverse transcriptase present therein is typically raised to 55 °C to 70 °C for 5 to 60 minutes, usually to about 65 °C for 15 to 20 minutes. Alternatively, reverse transcriptase may irreversibly inactivated by introducing a reagent into the reaction

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mixture that chemically alters the protein so that it no longer has RNA-dependent DNA polymerase activity. In yet other embodiments, the reverse transcriptase is reversibly inactivated. In these embodiments, the transcription may be carried out in the presence of an inhibitor of RNA-dependent DNA polymerase activity. Any

5 convenient reverse transcriptase inhibitor may be employed which is capable of inhibiting RNA-dependent DNA polymerase activity a sufficient amount to provide for linear amplification. However, these inhibitors should not adversely affect RNA polymerase activity. Reverse transcriptase inhibitors of interest include ddNTPs, such as ddATP, ddCTP, ddGTP or ddTTP, or a combination thereof, the total concentration

10 of the inhibitor typically ranges from about 50 μM to 200 μM.

For this transcription step, the presence of the RNA polymerase promoter region on the double-stranded cDNA is exploited for the production of antisense RNA. To synthesize the antisense RNA, the double-stranded DNA is contacted with the appropriate RNA polymerase in the presence of the four ribonucleotides, under

15 conditions sufficient for RNA transcription to occur, where the particular polymerase employed will be chosen based on the promoter region present in the double-stranded DNA, *e.g.* T7 RNA polymerase, T3 or SP6 RNA polymerases, *E. coli* RNA polymerase, and the like. Suitable conditions for RNA transcription using RNA polymerases are known in the art, see *e.g.* Milligan and Uhlenbeck (1989), Methods in

20 Enzymol. 180, 51. As mentioned above, a critical feature of the subject methods is that this transcription step is carried out in the presence of a reverse transcriptase that has been rendered inactive, *e.g.* by heat inactivation or by the presence of an inhibitor.

Because of the nature of the subject methods, all of the necessary polymerization reactions, *i.e.*, first strand cDNA synthesis, second strand cDNA

25 synthesis and antisense RNA transcription, may be carried out in the same reaction vessel at the same temperature, such that temperature cycling is not required. As such, the subject methods are particularly suited for automation, as the requisite reagents for each of the above steps need merely be added to the reaction mixture in the reaction vessel, without any complicated separation steps being performed, such as

30 phenol/chloroform extraction. A further feature of the subject invention is that, despite its simplicity, it yields high amplification extents, where the amplification extents (mass of RNA product/mass of RNA target) typically are at least about 50-fold, usually at least about 200-fold and may be as high as 600-fold or higher. Furthermore, such amplification extents are achieved with low variability, *e.g.*
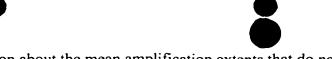
11

coefficients of variation about the mean amplification extents that do not exceed about 10%, and usually do not exceed about 5%.

The resultant antisense RNA produced by the subject methods finds use in a variety of applications. For example, the resultant antisense RNA can be used in expression profiling analysis on such platforms as DNA microarrays, for construction of "driver" for subtractive hybridization assays, for cDNA library construction, and the like. Especially facilitated by the subject methods are studies of differential gene expression in mammalian cells or cell populations. The cells may be from blood (*e.g.*, white cells, such as T or B cells) or from tissue derived from solid organs, such as brain, spleen, bone, heart, vascular, lung, kidney, liver, pituitary, endocrine glands, lymph node, dispersed primary cells, tumor cells, or the like. The RNA amplification technology can also be applied to improve methods of detecting and isolating nucleic acid sequences that vary in abundance among different populations using the technique known as subtractive hybridization. In such assays, two nucleic acid populations, one sense and the other antisense, are allowed to mix with one another with one population being present in molar excess ("driver"). Under appropriate conditions, the sequences represented in both populations form hybrids, whereas sequences present in only one population remains single-stranded. Thereafter, various well known techniques are used to separate the unhybridized molecules representing differentially expressed sequences. The amplification technology described herein may be used to construct large amounts of antisense RNA for use as "driver" in such experiments.

Depending on the particular intended use of the subject antisense RNA, the antisense RNA may be labeled. One way of labeling which may find use in the subject invention is isotopic labeling, in which one or more of the nucleotides is labeled with a radioactive label, such as ^{32}S, ^{32}P, ^{3}H, or the like. Another means of labeling is fluorescent labeling in which fluorescently tagged nucleotides, e.g. CTP, are incorporated into the antisense RNA product during the transcription step. Fluorescent moieties which may be used to tag nucleotides for producing labeled antisense RNA include: fluorescein, the cyanine dyes, such as Cy3, Cy5, Alexa 542, Bodipy 630/650, and the like. Other labels may also be employed as are known in the art.

Also provided are kits for use in the subject invention, where such kits may comprise containers, each with one or more of the various reagents (typically in

concentrated form) utilized in the methods, including, for example, buffers, the appropriate nucleotide triphosphates (*e.g.* dATP, dCTP, dGTP, dTTP, ATP, CTP, GTP and UTP), reverse transcriptase, RNA polymerase, and the promoter-primer of the present invention. Also present in kits according to the subject invention is a reverse transcriptase inhibitor, where in many embodiments, the inhibitor is at least one ddNTP or a combination of ddNTPs, *e.g.* ddATP and/or ddGTP. A set of instructions for use of kit components in an mRNA amplification method of the present invention, will also be typically included, where the instructions may be associated with a package insert and/or the packaging of the kit or the components thereof.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

I. MATERIALS AND METHODS, EXAMPLE 1

A. Reagents:

cDNA Synthesis Reaction

1. Poly-A RNA. Human HeLa cell poly-A RNA can be purchased from Clontech, P/N 6522-1

2. DNA T7T18VN (20 μM): (5') AAT TAA TAC GAC TCA CTA TAG GGA GAT TTT TTT TTT TTT TTT TTV N (3') (V = A/C/G, N = A/C/G/T) (SEQ ID NO:01)

3. MMLV Reverse Transcriptase (50 U/μl), Epicentre P/N M4425H

4. RNAGuard, Pharmacia P/N 27-0815-01

5. 5x First Strand Buffer: 250 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl, Life Technologies P/N 18057-018

6. 100 mM DTT, supplied with MMLV Reverse Transcriptase, Epicentre

7. dNTPs (10 mM each), diluted from Pharmacia P/N 2702035-01

8. Nuclease-free water, Amresco P/N E476

Transcription Reaction

1. T7 RNA Polymerase, Epicentre P/N TU950K

5 2. RNAGuard, Pharmacia P/N 27-0815-01

3. Inorganic Pyrophosphatase (200 U/ml), diluted from Sigma P/N I-1891

4. 5x Transcription Buffer: 0.2 M Tris-HCl, pH 7.5, 50 mM NaCl, 30 mM $MgCl_2$, 10
10 mM spermidine, Epicentre P/N BP001

5. 100 mM DTT, Epicentre P/N BP001

6. $MgCl_2$ (200 mM), diluted from Sigma P/N M-1028
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7. NTPs (25 mM ATP, GTP, UTP, 7.5 mM CTP), diluted from Pharmacia P/N 27-
2025-01

8. Cy3-CTP *OR* Cy5-CTP (7.0 mM)
20
9. Nuclease-free water, Amresco P/N E476

10. Lithium Chloride (4.0 M), diluted from Sigma P/N L-7026

25 11. 70% ethanol, diluted from Amresco E193

12. TE pH 8.0, Amresco P/N E112

30 **B.   Procedure:**

1. Add 100 ng to 600 ng poly-A RNA to reaction tube. Add 1.0 µl DNA T7T18VN
(20 µM) and bring total sample volume to 11.5 µl in nuclease-free water. Incubate
65 C for 10 min to denature primer and template. Quick chill on ice.
35
2. Mix the following components and maintain on ice. For more than one reaction,
multiply by the number of reactions.

cDNA Mix	
Component	Volume (µl)
5x First Strand Buffer	4.0
100 mM DTT	2.0
dNTPs (10 mM each)	1.0
MMLV-RT (50 U/µl)	1.0
RNAGuard (36 U/µl)	0.5
Volume of cDNA Mix	8.5

40 3. Aliquot 8.5 µl of cDNA Mix into each sample tube.
Incubate cDNA synthesis reaction at 40 C for 120 min.

14

Composition of cDNA Synthesis Reaction	
Component	Final concentration or amount
poly-A RNA	200 ng
DNA T7T18VN	1 μM
Tris-HCl, pH 8.3	50 mM
MgCl$_2$	3.0 mM
KCl	75 mM
DTT	10 mM
dNTPs	0.5 mM each
MMLV-RT	50 U
RNAGuard	18 U
Total reaction volume	20 μl

4. Incubate reaction tubes at 65 C for 15 min.
Move reaction tubes to ice. Store reactions tubes on ice for 5 min.

5. Immediately before use, mix the following components at room temperature. For more than one reaction, multiply by the number of reactions.

Transcription Mix	
Component	Volume (μl)
Nuclease-free water	20.8
5x Transcription Buffer	16
100 mM DTT	6.0
NTPs (25 mM A, G, U, 7.5 mM CTP)	8.0
Cy3-CTP (7.0 mM) *OR* Cy5-CTP (7.0 mM)	4.0
200 mM MgCl$_2$	3.3
RNAGuard (36 U/μl)	0.5
Inorganic Pyrophosphatase (200 U/ml)	0.6
T7 RNA polymerase (2500 U/μl)	0.8
Volume of Transcription Mix	60

6. Aliquot 60 μl of Transcription Mix into each sample tube.
Incubate transcription reactions at 40 C for 60 min.

Composition of Transcription Reaction	
Component	Final concentration or amount
Double-strand cDNA	Approximately 400 ng
Tris-HCl, pH 7.5	52 mM
MgCl$_2$	15 mM
KCl	19 mM
NaCl	10 mM
Spermidine	2 mM
DTT	10 mM
ATP, GTP, UTP	2.5 mM each

CTP	0.75 mM
Cy3-CTP *OR* Cy5-CTP	350 µM
T7 RNA polymerase	2000 U
RNAGuard	18 U
Inorganic pyrophosphatase	0.12 U
Total reaction volume	80 µl

7. Add 20 µl 50 mM EDTA to each reaction tube to stop the reaction. Alternatively, if quantitation of the amplified RNA product is desired, purifiy the RNA from unincorporated nucleotides by precipitation of the antisense RNA products in 2.0 M LiCl. Measure the RNA concentration by absorbance at OD_{260} using the conversion: 1 OD_{260} = 40 µg/ml RNA. Measure concentration of Cy3-CTP or Cy5-CTP by absorbance at OD_{552} (ε = 150 (1/mMcm)) or (ε = 250 (1/mMcm)), respectively.

II.   MATERIALS AND METHODS, EXAMPLE 2

A.   Reagents:

First Strand cDNA Synthesis

1. Poly-A RNA. Human HeLa cell poly-A RNA can be purchased from Clontech, P/N 6522-1

2. DNA T7T18VN (20 µM): (5') AAT TAA TAC GAC TCA CTA TAG GGA GAT TTT TTT TTT TTT TTT TTV N (3') (V = A/C/G, N = A/C/G/T) (SEQ ID NO:01)

3. Superscript II Reverse Transcriptase, Life Technologies P/N 18064-014

4. RNAGuard, Pharmacia P/N 27-0815-01

5. 5x First Strand Buffer*: 250 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 375 mM KCl
   * supplied with Superscript II Reverse Transcriptase, Life Technologies

6. 100 mM DTT, supplied with Superscript II Reverse Transcriptase, Life Technologies

7. dNTPs (10 mM each), diluted from Pharmacia P/N 2702035-01

8. Nuclease-free water, Amresco P/N E476

Second Strand cDNA Synthesis

1. MMLV Reverse Transcriptase, Epicentre P/N M4425H

2. 5x First Strand Buffer*: 250 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 375 mM KCl, supplied with Superscript II Reverse Transcriptase, Life Technologies

HP Ref: 10990638-1

3. dNTPs (10 mM each), diluted from Pharmacia P/N 2702035-01

4. Nuclease-free water, Amresco P/N E476

Transcription Reaction

1. T7 RNA Polymerase, Epicentre P/N TU950K

2. RNAGuard, Pharmacia P/N 27-0815-01

3. Inorganic Pyrophosphatase (200 U/ml), diluted from Sigma P/N I-1898

4. 5x Transcription Buffer: 0.2 M Tris-HCl, pH 7.5, 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine, Epicentre P/N BP001

5. 100 mM DTT, Epicentre P/N BP001

6. $MgCl_2$ (400 mM), diluted from Sigma P/N M-1028

7. NTPs (25 mM ATP, GTP, UTP, 7.5 mM CTP), diluted from Pharmacia P/N 27-2025-01

8. Cy3-CTP *OR* Cy5-CTP (7.0 mM)

9. ddATP (5 mM), Pharmacia P/N 27-2057-01

10. ddGTP (5 mM), Pharmacia P/N 27-2075-01
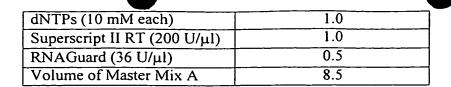
11. Nuclease-free water, Amresco P/N E476

12. Lithium Chloride (4.0 M), diluted from Sigma P/N L-7026

13. 70% ethanol, diluted from Amresco E193

14. TE pH 8.0, Amresco P/N E112

B.   Procedure:

1. Add 100 ng to 600 ng poly-A RNA to reaction tube. Add 1.0 µl DNA T7T18VN (20 µM) and bring total sample volume to 11.5 µl in nuclease-free water. Incubate 70 C for 10 min to denature primer and template. Quick chill on ice.

2. Mix the following components and maintain on ice. For more than one reaction, multiply by the number of reactions.

Master Mix A	
Component	Volume (µl)
5x First Strand Buffer	4.0
100 mM DTT	2.0

17

dNTPs (10 mM each)	1.0
Superscript II RT (200 U/μl)	1.0
RNAGuard (36 U/μl)	0.5
Volume of Master Mix A	8.5

3. Aliquot 8.5 μl of the master mix A into each sample tube. Incubate first strand synthesis reaction at 40 C for 60 min.

Composition of First Strand Synthesis Reaction	
Component	Final concentration or amount
poly-A RNA	200 ng
DNA T7T18VN	1 μM
Tris-HCl, pH 8.3	50 mM
$MgCl_2$	3 mM
KCl	75 mM
DTT	10 mM
dNTPs	0.5 mM each
Superscript II RT	200 U
RNAGuard	18 U
Total reaction volume	20 μl

4. Mix the following components and maintain on ice. For more than one reaction, multiply by the number of reactions.

Master Mix B	
Component	Volume (μl)
5x First Strand Buffer	4.0
dNTPs (10 mM each)	1.0
MMLV-RT (50 U/μl)	1.0
Nuclease-free water	13
Volume of master mix B	20

5. Aliquot 20 μl of the Master Mix B into each sample tube. Incubate second strand synthesis reaction at 40 C for 60 min.

Composition of Second Strand Synthesis Reaction	
Component	Final concentration or amount
cDNA	Approximately 200 ng
Tris-HCl, pH 8.3	50 mM
$MgCl_2$	3 mM
KCl	75 mM
DTT	5 mM
dNTPs	0.5 mM each
MMLV-RT	50 U
Total reaction volume	40 μl

HP Ref: 10990638-1

6. Immediately before use, mix the following components at room temperature. For more than one reaction, multiply by the number of reactions.

Master Mix C	
Component	Volume (µl)
Nuclease-free water	0.8
5x Transcription Buffer	16
100 mM DTT	6.0
NTPs (25 mM A, G, U, 7.5 mM CTP)	8.0
Cy3-CTP *OR* Cy5-CTP (7.0 mM)	4.0
400 mM MgCl$_2$	1.7
ddATP (5.0 mM)	0.8
ddGTP (5.0 mM)	0.8
RNAGuard (36 U/µl)	0.5
Inorganic Pyrophosphatase (200 U/µl)	0.6
T7 RNA polymerase (2500 U/µl)	0.8
Volume of Master Mix C	40

5   7. Aliquot 40 µl of the master mix C into each sample tube. Incubate transcription reactions at 40 C for 60 min.

Composition of Transcription Reaction	
Component	Final concentration or amount
Double-strand cDNA	Approximately 400 ng
Tris-HCl, pH 8.1	65 mM
MgCl$_2$	16 mM
KCl	37.5 mM
NaCl	10 mM
Spermidine	2 mM
DTT	10 mM
ATP, GTP, UTP	2.5 mM each
CTP	0.75 mM
Cy3-CTP *OR* Cy5-CTP	350 µM
ddATP, ddGTP	50 µM each
T7 RNA polymerase	2000 U
RNAGuard	18 U
Inorganic pyrophosphatase	0.12 U
Total reaction volume	80 µl

8. Add 20 µl 50 mM EDTA to each reaction tube to stop the reaction. Alternatively,
10   if quantitation of the amplified RNA product is desired, purifiy the RNA from unincorporated nucleotides by precipitation of the antisense RNA products in 2.0 M LiCl. Measure the RNA concentration by absorbance at OD_{260} using the conversion: 1 OD_{260} = 40 µg/ml RNA. Measure concentration of Cy3-CTP or Cy5-CTP by absorbance at OD_{552} (ε = 150 (1/mMcm)) or (ε = 250 (1/mMcm)),
15   respectively.

19

III. RESULTS

The above protocols provide two variations of the subject method for the amplification of heterogeneous mRNA populations. Both embodiments of the procedure perform essentially equivalently and yield amplification extents (mass of antisense RNA product/mass of mRNA target) up to 600-fold. The products of the amplification reaction are antisense RNA molecules with a size distribution resembling that of the cellular mRNA targets. The antisense RNA products accumulate linearly with respect to time, and thus bias in the relative amounts of specific RNA products associated with exponential amplification is not likely to occur. In fact, the pattern of hybridization intensities on cDNA arrays using fluorescently labeled amplified RNA is very similar to those obtained using labeled cDNA target.

Figure 1 shows that the amplification method described herein shows significantly higher performance than that described by U.S. Patent No. 5,716,785. The protocol describing a modification of the method described in this patent that was used for this analysis is found in Appendix A, *infra*. Amplification extents (micrograms of RNA product/micrograms RNA target) using the amplification method described in this disclosure were significantly higher (average = 300) with lower variability (coefficient of variation (CV) = 7.0%) than those using the method described by Van Gelder *et al.* (average = 69, CV = 35%).

The above results and discussion demonstrate that novel and improved methods of producing linearly amplified amounts of antisense RNA from an initial mRNA source are provided. The subject methods provide for an improvement over prior methods of producing antisense RNA in that the step of separating the double-stranded cDNA from the reverse transcriptase used for its preparation is not required. Instead, all of the steps for linearly amplifying mRNA into antisense RNA may by carried out in a single reaction mixture without performing a separation step. As such, the subject methods are amenable to automation, making them particularly attractive for high throughput applications. Furthermore, linear amplification extents of 600-fold and labeling efficiencies of 8% can be achieved using the subject methods. Finally, all of the benefits of linear amplification are achieved with the subject methods, such as the production of unbiased antisense RNA libraries from heterogeneous mRNA mixtures. As such, the subject methods represent a significant contribution to the art.

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All publications and patent application cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of

5   any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Although the foregoing invention has been described in some detail by way of

10 illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

HP Ref: 10990638-1